Phylogenetic placement of *Blastocladiopsis parva* (*Blastocladiomycota*)

William J. Davis
Department of Biological Sciences, The University of Alabama, Tuscaloosa, Alabama, USA
daviswj@umich.edu

and

Martha J. Powell
Department of Biological Sciences, The University of Alabama, Tuscaloosa, Alabama, USA

ABSTRACT

Blastocladiopsis parva was originally placed in *Blastocladia* under an invalid name, and was later segregated out of that genus based on resting spore morphology. Later authors showed resting spore morphology was not sufficient to delineate a new genus and advocated using physiology and growth form but did not formally amend the generic description. Using an isolate obtained in Alabama and amplicon based next generation sequencing, we place *Blastocladiopsis parva* in a molecular phylogeny, which confirms separating it from *Blastocladia* and emend the generic description to include growth form and physiology.Published on-line www.phytologia.org *Phytologia 100(1): 104-110 (Mar 16, 2018).* ISSN 030319430.

KEY WORDS: Ion Torrent, taxonomy, zoosporic fungi

Blastocladiomycota are an ecologically diverse zoosporic fungal lineage (Porter et al. 2011). A recent molecular phylogeny of the *Blastocladiomycota* revealed the non-monophyly of the family *Blastocladiaceae*, which contains the saprobic genera *Allomyces*, *Microallomyces*, *Blastocladia*, *Blastocladiella*, and *Blastocladiopsis* (Porter at el 2011). In the phylogeny of Porter et al. (2011), representatives of *Allomyces*, *Microallomyces*, and *Blastocladia* form a well-supported clade corresponding to the *Blastocladiaceae* while representatives of *Blastocladiella* group with representatives of *Catenariaceae* (Porter et al. 2011). Neither *Blastocladiopsis parva* (Whiffen) Sparrow, the type of *Blastocladiopsis*, or *Blastocladiopsis elegans* J. A. Robertson were included in the analysis (Porter et al. 2011), and their phylogenetic placement remains unknown.

Blastocladiopsis parva was originally described by Whiffen (1943) under the invalid name "*Blastocladia parva*". Whiffen failed to provide the required Latin description necessary in 1943 (McNeill et al. 2012), hence the binomial was invalidly published. The thallus of her fungus consisted of nonspetate, sub-dichotomously to dichotomously branched filaments with apical zoosporangia or resting spores (Whiffen 1943). It is saprobic in the soil and grows on snake skin and grass baits (Sparrow 1960). Sparrow (1950) reclassified Whiffen's fungus by placing it into the newly described genus *Blastocladiopsis* based on the resting spores being loose in the sporangia and having smooth walls, which contrasted with other members of *Blastocladia*. Sparrow (1950) provided a Latin description of the genus and included only one species, *B. parva*. By doing so, Sparrow validated both the generic name and the species binomial via a "description generic-specifica" as allowed by Art. 38.5 and as defined by Art. 38.6 (McNeill et al. 2012). Emerson and Robertson (1974) found resting spore characters to be insufficient to delineate genera within the *Blastocladiaceae* and suggested that generic boundaries be reformulated using physiology and growth form. They felt better features to distinguish *Blastocladiopsis* from *Blastocladia* were its growth on protein rich substrates (e.g., keratin) and the fact that "hyphal tips were not renewed by sympodial branching", i.e., growth along each branch was "strictly determinant" (Emerson and Robertson 1974). During a recent survey of chytrid diversity, we observed *Blastocladiopsis parva* in agricultural soils baited with snake skin, and sequenced the LSU rDNA region using next-generation sequencing. Herein, we report *Blastocladiopsis parva*'s placement within the phylogeny of Porter et al.

(2011) and emend the description of *Blastocladiopsis* to include Emerson and Robertson's (1974) suggestions.

MATERIALS AND METHODS

We collected soil from a ditch beside an agricultural field off Hwy 25 in Faunsdale, Alabama, USA (32.367241, −87.627883) in a sterile whirl-top bag and transported it on ice. In the laboratory, we placed a portion of the soil in a sterile Petri dish with sterile water and baited it with sterile snake skin. We photographed thalli of *Blastocladiopsis parva* using a Zeiss Axioskop with a Zeiss AxioCam MRc3 camera.

DNA extractions

We placed snake skin with month-old thalli of *Blastocladiopsis parva* in two sterile 2 mL tubes. To each tube, we added 200 µL of PL1 solution from a NucleoSpin Plant II DNA extraction kit (Macherey-Nagel Inc., Bethlehem, PA) and glass beads. We ground the samples with a mortar for 8 mins, added 10µl of RNase A and 10 µL Proteinase K and vortexed for 1 min. We then incubated the samples at 65C for 30 mins with brief (10s) vortexing every 15 mins. We transferred the samples to a 4C ice bath for 36 mins, and then incubated them for 5 mins at 65C. To each sample, we added 100 µL of chloroform and vortexed for 1 min. We then centrifuged the samples for 15 mins at 13k rpms. We transferred the aqueous layer to a sterile 2mL and brought the volume of the samples to the 0.75 mL line with sterile water. We then added 750 µL of phenol:chloroform to each samples. Samples were hand-shook vigorously for 3 mins and centrifuged for 5 mins at 13k rpms. The aqueous layer was transferred to a sterile 2mL tube and combined with 1mL of chloroform. The samples were hand-shook vigorously for 3mins and then centrifuged for 5 mins at 13k rpms. The aqueous layer was transferred to a new 2mL tube. We added 100 µL NaOAc and 1mL of isopropanol to each sample. Samples were incubated at −20C for 30mins before centrifuging for 15mins at 13k rpms. We discarded the supernant and added 500 µL of cold 75% ethanol. Samples were vortexed for 10 s and centrifuged for 3 mins at 13k rpms. The supernant was discarded and a second wash with 500 µL of cold 75% ethanol was performed. The supernant was discarded, and the tubes were dried under a laminar flow for 30 mins. The DNA pellet was re-suspended in 100 µL of TE buffer.

Amplicon generation & sequencing

DNA was amplified using fusion barcoded LROR (5'-ACCCGCTGAACTTAAGC-3' (Vilgalys and Hester 1990) and EDF360r (5'-TACTTGTICGCTATCGGTCTC-3'), a fungal affinity reverse primer targeting 300bp of the LSU rDNA region (Picard 2017). PCR amplifications were performed on 50ng of DNA under the following conditions: an initial denaturing step of 2 min at 94°C; 30 cycles of 1 min 94°C, 30 s 48°C, 15 s 51°C, and 1 min 72°C; and a final elongation step of 7 min at 72°C. Four replicate amplifications were pooled and dried in a 37°C incubator. The pellet was resuspended using 25µL of sterile water. Amplicons were gel purified via a NucleoSpin Extraction II kit (#740609.250; Macherey-Nagel, Inc.), quantified by fluorometry using a Qbit 2.0 (Invitrogen), and sent to Duke University for ion semiconductor (Ion Torrent) sequencing on a 314-chip using 400bp read chemistry (Sequencing and Genomic Technologies Shared Resource, Duke University, Durham, NC).

Analysis of next-generation amplicon data

The sample was run on the same chip as the samples in Davis et al. (in revision); thus, the initial processing was the same. Sequences were filtered based on a minimum average Phred quality score of 25 based on a sliding window of 50 bases, a size requirement of 200–350 bp, homopolymer run less than 6 bp, and a primer mismatch of 1 bp in QIIME 1.8.0 (Caporaso et al. 2010). Chimeras were detected with USEARCH v.6.1.544 (Edgar 2010) and the Ribosomal Database Project's 28S fungal database (No. 11; Liu et al. 2012) and removed in QIIME. OTUs were grouped at a 95% similarity threshold using uclust (Edgar 2010) in QIIME, and a representative set of sequences was generated using the most abundant

sequence belonging to an OTU. An OTU table was generated and filtered to include only the *Blastocaldiopsis parva* sample. This OTU table was used to pull the appropriate sequences from the representative sequences. Taxonomy was assigned using the Ribosomal Database Project's naïve-bayesian classifier 2.7 (RDB-NBC; Liu et al. 2012), which was retrained using a version of the RDP-NBC 28S fungal database No.11 that was hand curated to update the taxonomy of the chytrid sequences and to add additional chytrid sequences deposited in GenBank from taxonomic revisions and species descriptions. OTUs classified as Fungi at >75% support and Blastocladiomycota at >65% support were selected for phylogenetic analysis. Raw sequences are submitted to the NCBI Sequence Read Archive (PRJNA395910). Sequences representing the OTUs were submitted to GenBank (OTU 1284: MG925664; OTU 2114: MG925663). All scripts are deposited on GitHub (https://github.com/wjdavis90/Blastocladioposis_parva).

Phylogenetic analysis

The complete alignment of Porter et al. (2011) was downloaded from Treebase (treebase.org). The OTU sequences were added to the alignment using the 'add fragments' function of mafft v. 7.294 (Katoh et al. 2002; Katoh and Frith 2012; Katoh and Standley 2013). The alignment was filtered using Gblocks v. 0.91b (Castresana 200) with the minimum number of sequences for conserved and flanking positions set to 38, the maximum number of contiguous nonconserved positions set to 8, the minimum length of a block set to 5, and gaps allowed. Maximum likelihood (ML) trees were inferred using the parallel version of RAxML 8 (Stamatakis 2014). Trees were inferred using the GTR + GAMMA model of sequence evolution and bootstrapped with 1000 replicates.

RESULTS

The morphology of our isolate matched the description given by Whiffen (1943) and Sparrow (1950). Thalli exhibited determinant, dichotomously branching growth with terminal resting spores (Fig. 1A). Resting spores were detached from the sporangial wall and had smooth walls (Fig. 1B). Zoosporangia were not observed.

There were 18590 sequences to start. After quality control, there were 5893 sequences that grouped into 78 OTUs. Fungi was the most abundant taxon (66%) followed by Stramenopiles (26%) and Rhizaria (3%). Only OTUS 1284 and 2114 from the sample were identified as members of *Blastocladiomycota*.

There were 15689 positions in the original alignment, and Gblocks reduced it to 2946 positions (18%). The inferred tree had low bootstrap support (<50%) at multiple nodes, especially compared to the tree presented in Porter et al. (2011). Thus, we inferred a new phylogeny using all of the sites. The phylogeny inferred from the Gblocks selected positions is available on GitHub. In the phylogeny inferred from the full alignment, OTUs 1284 and 2114 are sister to a clade containing *Blastocladiella britainnica*, *Blastocladiella* sp. JEL363, *Blastocladiella* sp. JEL440 and strains of *Catenaria* with 85% bootstrap support (Fig. 2). *Blastocladia pringsheimii*, the type of the genus, forms a well-supported clade (88% bootstrap support) with representatives of *Allomyces* and *Microallomyces* in the *Blastocladiaceae* (Fig. 2).

TAXONOMY

Blastocladiopsis Sparrow, J. Wash. Acad. Sci. 40: 52. 1950 emend. W. J. Davis [this publication]
MycoBank 20080

Typification: *Blastocladiopsis parva* Sparrow

Etymology: *Blastocladi-* referring to the genus *Blastocladia*; *-opsis* from the Greek *opsis*= appearance.

Description: THALLUS on protein rich substrates filamentous, sub-dichotomously to dichotomously branched. RHIZOIDS course and branched. GROWTH determinant, sympodial branching after development of sporangia absent. ZOOSPORANGIA infrequent, irregular to cylindrical, borne singly with one to several discharge papillae. RESTING SPORES borne singly, held loosely within sporangia, gold, amber or dark brown with smooth to reticulate think walls and one to several discharge pores or papillae upon germination. ZOOSPORES posteriorly uniflagellate with several lipid globules and a nuclear cap. Differs from *Blastocladia* by growing as an obligate aerobe. Differs from *Blastocladia* and *Blastocladiella* by branching subdichotomously to dichotomously. Differs from *Allomyces* by showing strictly determinant growth; no sympodial branching after the formation of sporangia.

Blastocladiopsis parva Sparrow, J. Wash. Acad. Sci. 40: 52. 1950
MycoBank 293721
"*Blastocladia parva*" Whiffen, J. Elisha Mitchell Sci. Soc. 59: 40. 1943 [nom invalid.]

Typification: Soil from Terrell, Texas, USA. (**Holotype**-Fig. 17, Whiffen J. Elisha Mitchell Sci. Soc. 1943)

Etymology: from the Latin *parvus* = small, little

Description: English description provided under the name "*Blastocladia parva*" Whiffen [in 1943 a nom. invalid., Melbourne Code Art. 39.1]

THALLUS, as described by Whiffen (1943) and again by Sparrow (1950), 300µm or more in length, 12–50µm in diameter, subdichotomously to dichotomously branched. ZOOSPORANGIA infrequent, irregular to cylindrical with one to six discharge papillae. RESTING SPORES spherical to ellipsoid, gold to amber-colored, smooth-walled, lying loosely in sporangium with one to two discharge pores upon germination. ZOOSPORES posteriorly uniflagellate. Differs from *Blastocladiopsis elegans* by the smooth resting sporangia walls.

Additional specimens examined: USA, ALABAMA: Faunsdale, Hwy25, roadside ditch beside an agricultural field. Snake skin, soil. GenBank MG925664 (28s, OTU 1284) and MG925663 (28s, OTU 2114).

DISCUSSION

Sparrow (1950) described *Blastocladiopsis* distinguishing it from members of *Blastocladia* based on the space separating the thick wall of the resting spore from the thin wall of the sporangium and the smooth nature of the resting spore walls. Emerson and Robertson (1974) reviewed several species of the *Blastocladiaceae* and found these to be a common character states in the family. They proposed that the genera be delineated based on physiology and growth form (Emerson and Robertson 1974). They suggested the main character state separating *Blastocladiopsis* from *Blastocladia* was obligate aerobic growth on protein-rich substrates, and the main character state separating it from *Blastocladiella* was its branched growth form (Emerson and Robertson 1974). They concluded the fundamental character state defining this genus was that "the branches of *Blastocladiopsis* do not have the capacity for renewed growth by sympodial branching once they have formed sporangia at their apices" (Emerson and Robertson 1974, p. 314). Following Emerson and Robertson's (1974) concept of the genus, Robertson (1976) described *Blastocladiopsis elegans* even though the species had reticulate resting spores that essentially filled the sporangia. Thus, we emend Sparrow's (1950) description to incorporate and emphasize the physiology and growth form of this genus and de-emphasize the formation and structure of the resting spores following Emerson and Robertson (1974) and Robertson (1976). Our molecular phylogeny places *B. parva* in a clade distinct from the type of *Blastocladia*, which validates Sparrow's erection of a new genus for it. In our molecular phylogeny, *Blastocladiopsis parva* is sister to strains representing *Blastocladiella*. Since *Blastocladiopsis* differs morphologically from *Blastocladiella* by its branched growth form (Emerson and Robertson 1974), *Blastocladiella* is currently non-monophyletic, and neither the type species of *Blastocladiella* or *Blastocladiopsis elegans* are represented in the current

molecular phylogeny (Porter et al. 2011), we feel it is appropriate to retain *Blastocladiopsis* as its own genus until the placement of *Blastocladiella simplex* and *Blastocladiopsis elegans* is known.

ACKNOWLEDGMENTS

We express our gratitude to Scotty DePriest and Trey Melton for aid in the collection of the soil; Kathryn Picard for help with the amplification, sequencing, and analysis of the amplicons; Satoshi Sekimoto for help with the DNA extraction; Janna Fierst, Chamali Thanthiriwatte, and Daryl Lam for help with the bioinformatic problems we frequently encountered; and Rabern Simmons for proofreading the manuscript. We are grateful to the reviewers Scott A. Redhead and Terri M. Porter for their help in making the manuscript better.

LITERATURE CITED

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. Gonzalez Pena, J. K. Goodrich, J. I. Gordon, G.A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W.A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, and R. Knight. 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7: 335-336. https://dx.doi/10.1038/nmeth.f.303

Castresana, J. 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analyses. Molecular Biology and Evolution 17: 540–552.

Davis, W. J., K. Picard, J. Antonetti, J. Edmonds, J. Fults, P. M. Letcher, and M. J. Powell. In Revision. Inventory of chytrid diversity in two temporary forest ponds using a multiphasic approach. Mycologia.

Edgar, R. C. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460-2461. https://doi.org/10.1093/bioinformatics/btq461

Emerson R, JA Robertson. 1974. Two new members of the *Blastocladiaceae*. I. Taxonomy, with an evaluation of genera and interrelationships in the family. American Journal of Botany 61: 303–317.

Liu, K.L., A. Porras-Alfaro, C. R. Kuske, S. A. Eichorst, and G. Xie. 2012. Accurate and rapid taxonomic classification of fungal large-subunit rRNA genes. Applied and Environmental Microbiology 78:1523–1533. https://dx.doi/10.1128/AEM.06826-11

Katoh, K., K. Misawa, K-i. Kuma, and T. Miyata. 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Forier transform. Nucleic Acids Research 30:3059–3066. https://doi.org/10.1093/nar/gkf436

Katoh, K. and M. C. Frith. 2012. Adding unaligned sequences into an existing alignment using MAFFT and LAST. Bioinformatics 28:3144–3146. https://doi.org/10.1093/bioinformatics/bts578

Katoh K. and D. M. Standley. 2013. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in performance and usability. Molecular Biology and Evolution 30:772–780. https://doi.org/10.1093/molbev/mst010

McNeill J, FR Barrie, WR Buck, V Demoulin, W Greuter, DL Hawksworth. PS Heredeen, S Knapp, K Marhold, J Prado, WF Prud'homme Van Reine, GF Smith, JH Wiersema. 2012. International code of nomenclature for algae, fungi, and plants (Melbourne Code). Regnum Vegetabile 154. Koeltz Scientific Books.

Picard, K. T. 2017. Coastal marine habitats harbor novel early-diverging fungal diversity. Fungal Ecology 25:1–13. https://doi.org/10.1016/j.funeco.2016.10.006

Porter TM, W Martin, TY James, JE Longcore, FH Gleason, PH Adler, PM Letcher, R Vilgalys. Molecular phylogeny of the *Blastocladiomycota* (Fungi) based on nuclear ribosomal DNA. Fungal Biology 115: 381–392. https://doi.org/10.1016/j.funbio.2011.02.004

Robertson JA. 1976. *Blastocladiopsis elegans*, a new member of the *Blastocladiales*. Canadian Journal of Botany 54: 611–615. https://dx.doi/ 10.1139/b76-063

Sparrow FK 1950. Some Cuban phycomycetes. Journal of the Washington Academy of Sciences 40: 50–55.

Sparrow, F.K. 1960. Aquatic phycomycetes. 2nd ed. University of Michigan Press, Ann Arbor, Michigan. 1187 pp.
Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30: 1312–1313. https://doi.org/10.1093/bioinformatics/btu033
Vilgalys, R. and M. Hester. 1990. Rapid identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. Journal of Bacteriology 172:4238–4246. https://dx.doi/ 10.1128/jb.172.8.4238-4246.1990
Whiffen AJ. 1943. A new species of *Nowakowskiella* and *Blastocladia*. Journal of the Elisha Mitchell Scientific Society 59: 37–43.

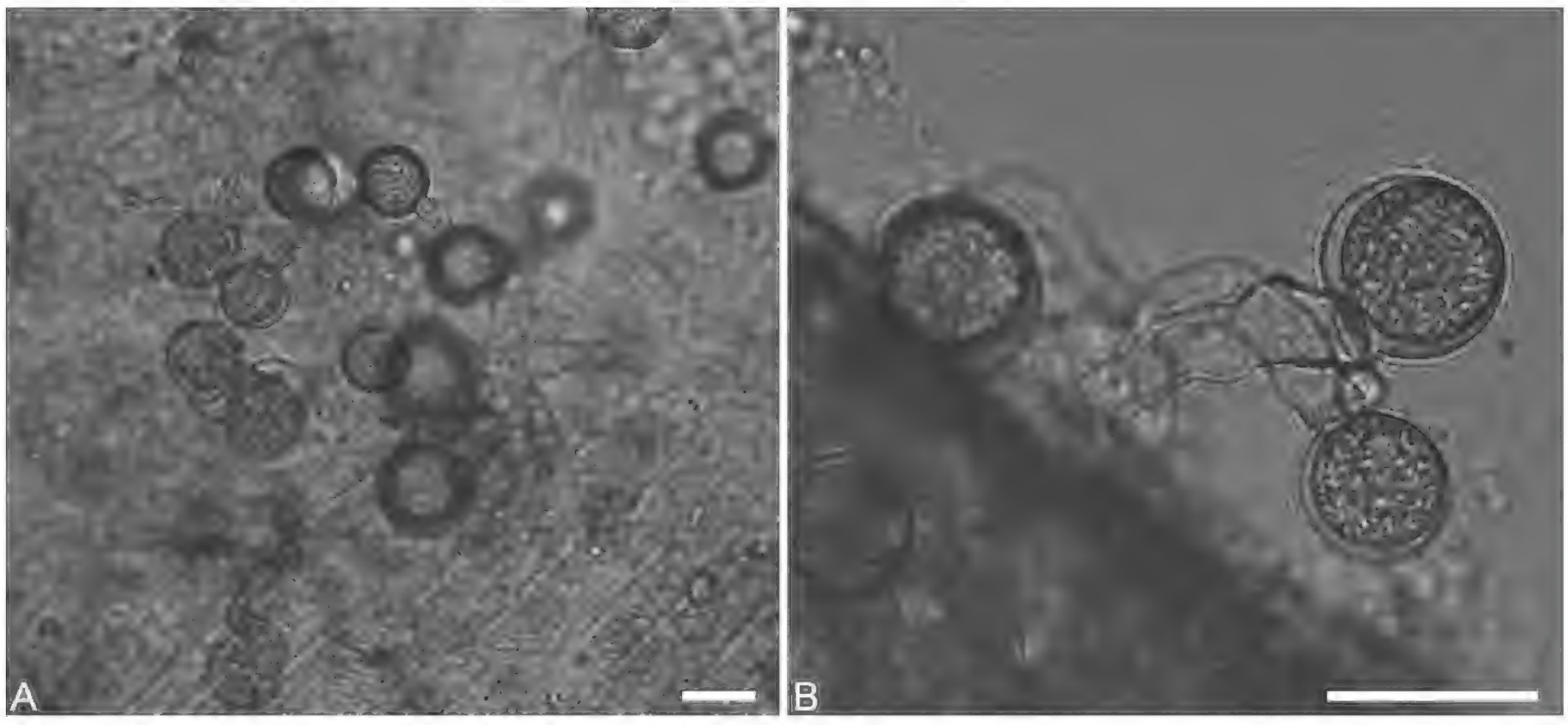

Figure 1. Light micrographs of *Blastocladopsis parva*. A. Dichotomously branched thallus with multiple resting spores. Scale bar = 30µm. B. Close up of resting spores showing the smooth wall and detachment from the sporangium wall. Scale bar =30µm.

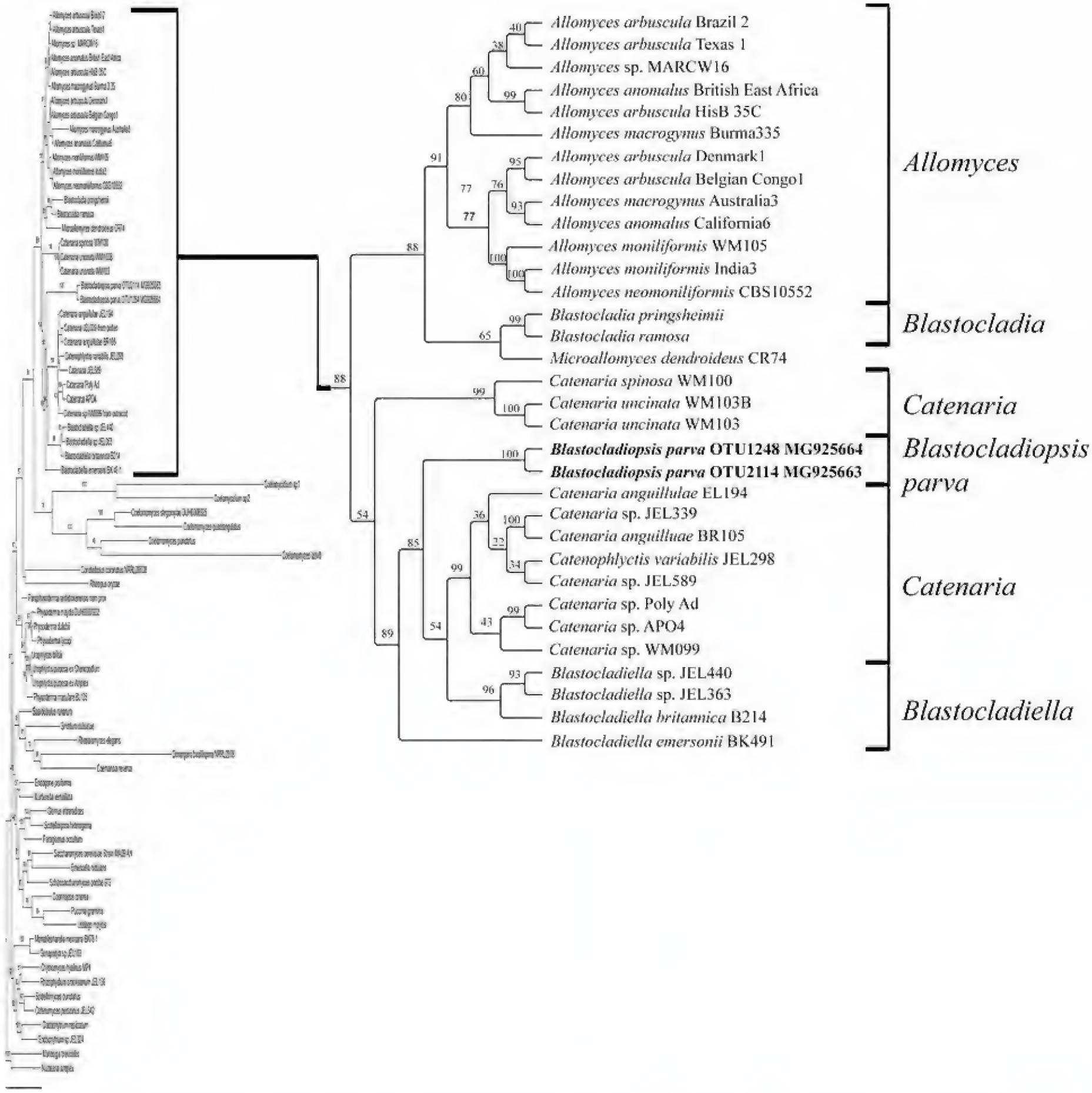

Figure 2. Inferred maximum likelihood phylogeny for *Blastocladiomycota*. The analysis included 71 sequences and 15689 positions. The full phylogeny is shown on the left and is rooted with *Monosiga brevicolis* and *Nuclearia simplex*; on the right, the *Blastocladiaceae* and *Catenariaceae* are shown as a cladogram. Bar = 1.0 changes per site.